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MARC-145 细胞 DMEM 培养基
使用手册 2008

北京清大天一生物技术有限公司

BEIJING TSINGHUA SKYWING BIO TECH Co.,LTD.

目 录

MARC-145 细胞DMEM培养基简介 1

MARC-145 细胞DMEM培养基使用范围 1

MARC-145 细胞DMEM培养基使用特点 2

MARC-145 细胞DMEM培养基使用效果 2

MARC-145 细胞DMEM培养基配制程序 3

MARC-145 细胞DMEM培养基配制注意事项 3

MARC-145 细胞DMEM培养基转瓶培养MARC-145 细胞程序 4

MARC-145 细胞DMEM培养基转瓶培养MARC-145 细胞注意事项 5

MARC-145 细胞DMEM培养基转瓶培养细胞生产蓝耳病疫苗的建议 5

微载体反应器培养MARC-145 细胞简介 5

附录 1. MARC-145 细胞DMEM培养基使用说明书 7

附录 2. MARC-145 细胞DMEM培养基pH/渗透压对照表 9

附录 3. 参考文献 10

牛血清在细胞培养中的作用及产生的常见问题 10

Porcine reproductive and respiratory syndrome virus (PRRSV) infection spreads by cell-to-cell transfer in cultured MARC-145 cells, is dependent on an intact cytoskeleton, and is suppressed by drug-targeting of cell permissiveness to virus infection 15

MARC-145 细胞 DMEM 培养基简介

MARC-145 细胞 DMEM 培养基（产品序号：MD210 及 MD900）是北京清大天一生物技术有限公司为MARC-145 细胞培养设计开发的个性化细胞培养基，可支持 MARC-145 细胞的快速生长，细胞传代分种比例和生长状况良好。另外 MD900 培养基可实现微载体反应器低血清培养 MARC-145 细胞，血清含量可降低到 5%左右。

MARC-145 细胞 DMEM 培养基是基于传统 DMEM 培养基配方的改良培养基，通过调整培养基中主要营养成分的种类、比例、含量，支持 MARC-145 细胞转瓶及微载体反应器低血清培养。该 DMEM 培养基配方成分中，不含任何血清替代物、动物来源蛋白、植物蛋白，具有良好的使用安全性。

使用 MD210 DMEM 培养基培养 MARC-145 细胞，细胞传代分种率可达到 1:3～1:4，细胞生长形态及状态良好。MD900 DMEM 培养基可用于 MARC-145 细胞微载体反应器低血清培养，有益于减少血清成分以及血清中潜在外源病毒对产品质量的危害，可提高产品质量；使用反应器微载体可提高产品生产效率，并提高病毒产率，可降低产品生产成本。

MARC-145 细胞 DMEM 培养基为粉末培养基，包装规格为 50L/包。与传统的 DMEM 培养基比较，配制使用方法基本相同，使用方法简单方便。

MARC-145 细胞 DMEM 培养基的生产、检验严格执行 GMP 和 ISO9001 管理体系，产品质量符合国家化工行业标准《哺乳类动物细胞培养基》要求，产品质量稳定可靠。

MARC-145 细胞 DMEM 培养基使用范围

MARC-145 细胞 DMEM 培养基为 MARC-145 细胞培养的专用个性化培养基。

MARC-145 细胞 DMEM 培养基可用于猪蓝耳病疫苗生产中 MARC-145 细胞制备，MARC-145 细胞可使用方瓶、3L 转瓶、15L 转瓶以及微载体反应器培养。与传统 DMEM 培养基比较，使用 MD210 培养基培养 MARC-145 细胞，可提高细胞传代分种比例、减少细胞培养时间、提高 MARC-145 细胞活力，适用于 MARC-145 细胞建库、种细胞复苏传代、以及细胞生产制备；使用 MD900 培养基微载体反应器低血清培养 MARC-145 细胞，在降低血清使用量的情况下，可提高生产效率。

MARC-145 细胞DMEM培养基可用于猪蓝耳病疫苗生产中猪繁殖与呼吸综合征病毒制备，用MD210 DMEM培养基替代传统的PRRSV病毒培养维持液（基础DMEM），可提高猪繁殖与呼吸综合征病毒滴度，适用于猪繁殖与呼吸综合征病毒种毒制备，以及病毒培养液的生产。

MD900 DMEM 培养基支持高密度 MARC-145 细胞微载体反应器培养，细胞生长良好。

MARC-145 细胞 DMEM 培养基可用于各种基于 MARC-145 细胞的科研和检验，用 MD210 DMEM 培养基培养制备 MARc-145 细胞，细胞具有生长旺盛、活力强等优点，有利于提高科研和检验工作的方便性和可靠性。

MARC-145 细胞 DMEM 培养基使用特点

MD210 及 MD900 DMEM 培养基为 MARC-145 细胞低血清培养的专用个性化培养基，其培养 MARC-145 细胞具有以下特点：

- 不含血清替代物、动物来源蛋白、以及植物蛋白、酵母水解物，具有使用安全性。
- 细胞生长旺盛，生长速度快。
- 细胞培养形态健壮、稳定，细胞活力强。
- 具有较高分种比率，方瓶静止培养分种比例可达 1:5～1:6，转瓶培养分种比例可达 1:3～1:4。
- 细胞易培养，可提高细胞培养的稳定性。
- 提高病毒产率和产量。
- 支持低血清培养，血清含量可降低到 5％左右，降低了培养液成本。
- 减少血清使用量，降低接种副反应，提高产品质量。

MARC-145 细胞 DMEM 培养基使用效果

- **MD210 DMEM 培养基方瓶培养 MARC-145 细胞**

使用 MD210 DMEM 培养基，在 T75 细胞培养瓶中培养 MARC-145 细胞，细胞轮廓清晰并具有较强立体感，形态良好，细胞生长旺盛，可以大比例分种进行细胞传代培养。以 1:5 分种比例进行细胞传代培养，培养 48～72 小时，细胞生长为单层或致密单层。



A

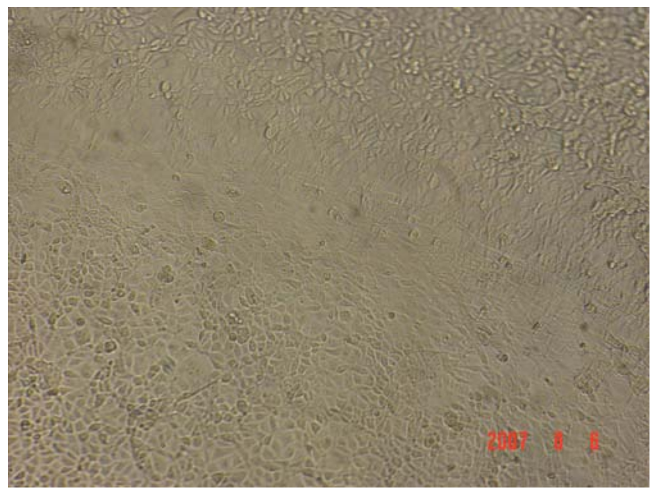


B

A. T75方瓶中培养MARC-145细胞, MD210+5%血清，72小时细胞长成致密单层
B. 更换培养液为MD210+2%血清，生长至第8天，细胞仍具极高活力状态

➢ MD210 DMEM 培养基转瓶培养 MARC-145 细胞生产猪蓝耳病疫苗

以 MD210 DMEM 培养基培养 MARC-1451 细胞生产猪蓝耳病疫苗，MARC-145 细胞可连续传代，培养细胞生长数量较传统 DMEM 培养基多，48h～72h 1:3～1:4 细胞能够稳定传代、生长，细胞致密，形态立体感较强。



MD210+8%NBS 转瓶 48h

MARC-145 细胞 DMEM 培养基配制程序

以 50L/包规格 MD210 DMEM 培养基为例，应按以下程序配制培养基:

1. 打开培养基包装，将培养基全部倒入一容器中。
2. 用少量冷却注射用水（20℃～30℃）涮洗袋内残留培养基，倒入容器内。
3. 向容器中加入约 40L 冷却注射用水（20℃～30℃），搅拌至培养基完全溶解。
4. 按使用说明书标示量加入 85 克碳酸氢钠，搅拌至完全溶解，补加冷却注射用水（20℃～30℃）至 50L，充分混合，用 pH 计检测，溶液 pH 应为 7.1～7.2。
5. 按使用量加入血清和抗生素，混合均匀，用 0.22μm 滤膜正压过滤除菌。
6. 除菌后培养基置 2℃～8℃避光保存，应在一周内使用。

MD900 培养基的配制过程同上。

MARC-145 细胞 DMEM 培养基配制注意事项

- ☐ 不可配制成浓缩液储存使用。
- ☐ 不可用高压湿热法进行灭菌。
- ☐ 不可冷冻储存，溶解使用。

- 不可常温储存，2～8℃储存应不超过 1 周时间。
- 该培养基中已含谷氨酰胺，由于谷氨酰胺不稳定，易分解，产生 MARC-145 细胞敏感的毒性物质，故加入血清的完全培养液应尽快使用完毕。
- 按标示量加入碳酸氢钠 pH 应为 7.1～7.2，过滤后 pH 可升高 0.1～0.2；如用开放式大罐长时间搅拌配液，可导致 pH 升高；如使用容器长时间储存，也可能导致 pH 升高。应注意不同配液方式对培养液 pH 的影响。
- 该培养基中所含指示剂酚红的量是常规量的一半，故从外观上看所配溶液颜色较浅，精确判定 pH 时，建议用 pH 计测定 pH。
- 按标示量加入碳酸氢钠，如达不到所需 pH 要求，应用 1N 氢氧化钠调节到所要求 pH 范围。如用碳酸氢钠调 pH 至 7.6 以上，应注意培养液渗透压的增加，可能影响细胞正常培养。

MARC-145 细胞 DMEM 培养基转瓶培养 MARC-145 细胞程序

传代种细胞选择标准

应选择形态良好、正常健康、分布均匀的转瓶细胞。
应选择形成单层或致密单层的处于对数生长期的转瓶细胞。

细胞消化

细胞消化液为含 0.25％胰酶和 0.025％EDTA 消化液，建议使用 Hanks'液或 PBS 溶液配制，pH 为 7.6～7.8。细胞消化液配制后应及时使用。
将细胞生长良好的培养瓶，轻微摇瓶后倒去细胞培养液，加入适量细胞消化液，转动细胞瓶使细胞消化液浸润细胞 1～3 分钟，倒去细胞消化液，静止细胞瓶待细胞滑落，加入细胞培养液，摇瓶分散细胞。

细胞分种、细胞培养

- 按 1:3～1:4 分种比例分种细胞，选择合适的分种比例。
- 15L 转瓶细胞培养温度为 37±0.5℃，转数 9～14rpm。
- 细胞培养时间为 2～3 天，细胞应生长为单层或致密单层。
- 细胞培养液使用血清含量为 5～10％。
- 15L 转瓶细胞培养液加量为 1.5～2 L。
- 当细胞生长为致密单层后应及时传代。

MARC-145 细胞 DMEM 培养基转瓶培养 MARC-145 细胞注意事项

- 用于细胞消化的细胞消化液，应现配现用，或冻存使用，应注意消化液细胞消化效果，充分消化细胞。
- 细胞培养阶段的血清浓度为 5～10%，由于国内血清质量差异，建议使用前进行细胞培养筛选，并建议实际使用血清浓度不低于 3%。
- 消化完成后加入足量的培养液充分摇匀分散细胞，必要时可吹打细胞使之分散。
- 用水平仪调整转瓶机的水平位置，使细胞能够在转瓶中均匀贴壁。
- 必要时可适当增加转瓶机的转速，使细胞均匀贴壁。

MARC-145 细胞 DMEM 培养基转瓶培养细胞生产蓝耳病疫苗的建议

病毒维持液 pH 值调节至 7.6。

病毒维持液血清浓度 2%，视血清质量，还可考虑降低血清浓度。

接种蓝耳病病毒后，建议做病毒增殖曲线，选定合适的收毒时间。

接种蓝耳病病毒，建议做不同接毒量的实验研究，即选择合适的病毒感染复数（MOI），可参阅附件的文献。

微载体反应器培养 MARC-145 细胞简介

微载体的预处理和生物反应器灭菌

称取适量的微载体，用不含钙镁离子的 PBS 溶液洗涤、浸泡后，与生物反应器一起进行灭菌。

MARC-145 的微载体培养

消化转瓶 MARC-145 细胞，接种到生物反应器中，设定并控制生物反应器一定的 pH、DO、搅拌速度和温度，进行细胞培养。

蓝耳病病毒的接种和维持

待生物反应器中的 MARC-145 细胞生长至合适密度，进行病毒维持液的更换，并按一定的感染复数接种蓝耳病病毒，进行病毒繁殖和收获。

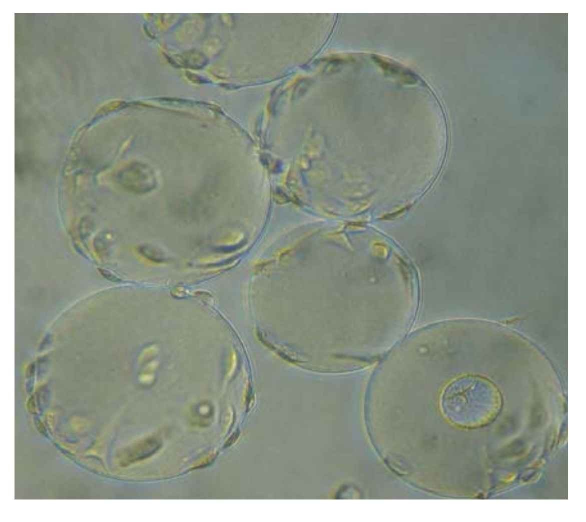
反应器培养 MARC-145 细胞实验

生物反应器：Clavorus THR 50L 和 Clavorus THR 100L

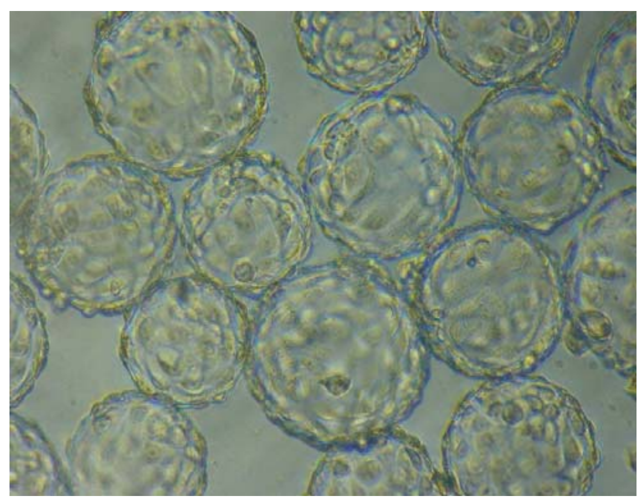
细胞培养基：清大天一反应器高密度 MARC145 细胞培养基（MD900）

微载体：GE Cytodex-1，5g/L

结果：MARC-145 细胞的密度可达 3.4～4×10^6cells/mL。接种后 24hr和 72hr的照片如下图。



微载体上生长 24hr 时的 MARC-145 细胞



微载体上生长 72hr 时的 MARC-145 细胞

微载体和转瓶培养 MARC-145 细胞的前景分析

培养体积为 75L 的反应器使用微载体培养 MARC-145 细胞，在微载体浓度为 5g/L 时所获得的细胞数相当于 400 个 15L 转瓶。如果提高微载体浓度，细胞培养的密度也能进一步提高。

因此使用生物反应器培养 MARC-145 细胞，能够实现工艺稳定的细胞高密度培养，减少能源、人力、厂房设备固定投资等成本，并且能迅速扩大生产规模。

附录 1. MARC-145 细胞 DMEM 培养基使用说明书

（一） MD210 DMEM 培养基使用说明书

产品代号：MD210-050　　每升标示量：12.48g　　标准编号：HG/T 3935-2007

一.【组成成分】

无机盐：氯化钠、氯化钙、无水硫酸镁、无水磷酸二氢钠、磷酸氢二钠等。

氨基酸：L-盐酸精氨酸、L-盐酸胱氨酸、L-谷氨酰胺、L-盐酸赖氨酸、L-甲硫氨酸、L-苯丙氨酸、L-苏氨酸、L-色氨酸、L-酪氨酸、L-缬氨酸等。

维生素：泛酸钙、维生素B_1、维生素B_2、维生素C、维生素H、盐酸吡哆醇、盐酸吡哆醛等。

其　它：葡萄糖、丙酮酸钠等。

二.【产品指标】

性状		粉红色固体粉末
澄清度		澄清
pH 值	①加$NaHCO_3$	6.80～7.40
	②不加$NaHCO_3$	5.20～5.80
干燥减量的质量分数（%）		≤5.0
渗透压（mOsm/kgH_2O）	①加$NaHCO_3$	282～312
	②不加$NaHCO_3$	248～274
细菌内毒素（EU/ml）		＜10
微生物限度检查(CFU/g)	≤200	≤200
	≤50	≤50
细胞生长试验	细胞形态	成纤维贴壁生长，形态正常无变异
	细胞生长数量	72h细胞数量＞1×10^5个/ml。继续维持 48h,细胞数量应≥1×10^5个/ml。
注：详细产品技术指标见质量标准		

三.【配制方法】

(1) 将一袋培养基全部倒入一容器中，用少量注射用水将袋内残留培养基洗下，并入容器。加注射用水（水温 20℃～30℃）到 47.5 升，轻微搅拌溶解。
(2) 加入 85 克碳酸氢钠。
(3) 轻微搅拌溶解，加注射用水至 50 升。
(4) 如果必要，用 1mol/L 氢氧化钠溶液或 1mol/L 盐酸溶液调 pH 至所需值。
(5) 用 0.2μm 滤膜正压过滤除菌。
(6) 溶液应在 2℃-8℃下避光保存。

四.【贮藏】　2℃-8℃冷藏，干燥、密封、避光贮藏。

五.【规格】　50L/包

六.【有效期】　二年

生产企业：　北京清大天一生物技术有限公司
地　　址：　北京昌平科技园区白浮泉路 11 号

http://www.cellculture.com.cn　http://www.tsinghuaty.com

电　　话：　010-80110922　　80110683

（二）MD900 DMEM 培养基使用说明书

产品代号：　MD900-050　　　每升标示量：12.5g

一.【主要成分】

无机盐：氯化钠、氯化钙、氯化钾、无水硫酸镁、无水磷酸二氢钠、无水磷酸氢二钠等。

氨基酸：L-盐酸精氨酸、L-盐酸赖氨酸、L－谷氨酸、L-谷氨酰胺、L－组氨酸盐酸盐、L-异亮氨酸、L-亮氨酸、L-脯氨酸、L-丝氨酸、L-苏氨酸、L-色氨酸等。

维生素：维生素 B1、维生素 B2、维生素 B6、维生素 B12、维生素 C、D-生物素、泛酸钙等。

其　它：无水葡萄糖、丙酮酸钠、次黄嘌呤、亚油酸、硫辛酸等。

二.【产品指标】

性状		类白色固体粉末
澄清度		澄清
pH 值	①加$NaHCO_3$	6.85～7.45
	②不加$NaHCO_3$	5.30～5.90
干燥减量的质量分数（%）		≤5.0
渗透压（mOsm/kgH_2O）	①加$NaHCO_3$	285～315
	②不加$NaHCO_3$	251～278
细菌内毒素（EU/ml）		＜10
微生物限度检查（CFU/g）	细菌数	≤200
	霉菌数	≤50
细胞生长试验	细胞形态	成纤维贴壁生长，形态正常无变异
	细胞数量	72h细胞数量＞1×10^5个/ml，继续维持 48h，细胞数量≥1×10^5个/ml
注：详细产品技术指标见质量标准		

三.【使用方法】

1. 将一袋培养基全部倒入一容器中，用少量注射用水将袋内残留培养基洗下，并入容器。加注射用水（水温 25℃～30℃）到 47.5 升，轻微搅拌溶解。
2. 加入 100 克碳酸氢钠。
3. 轻微搅拌溶解，加注射用水至 50 升。
4. 用 1mol / L 氢氧化钠溶液或 1mol / L 盐酸溶液调 pH 至所需值。
5. 用 0.2μm 滤膜正压过滤除菌。
6. 溶液应在 2℃－8℃下避光保存。

四.【贮藏】　　　2℃－8℃冷藏，干燥、密封、避光贮藏。

五.【规格】　　　50L/包

六.【有效期】　　二年

备注：

1. 产品含 L-谷氨酰胺，不含酚红。

2. 本产品成分为化学结构明确的物质，无动物来源物质。

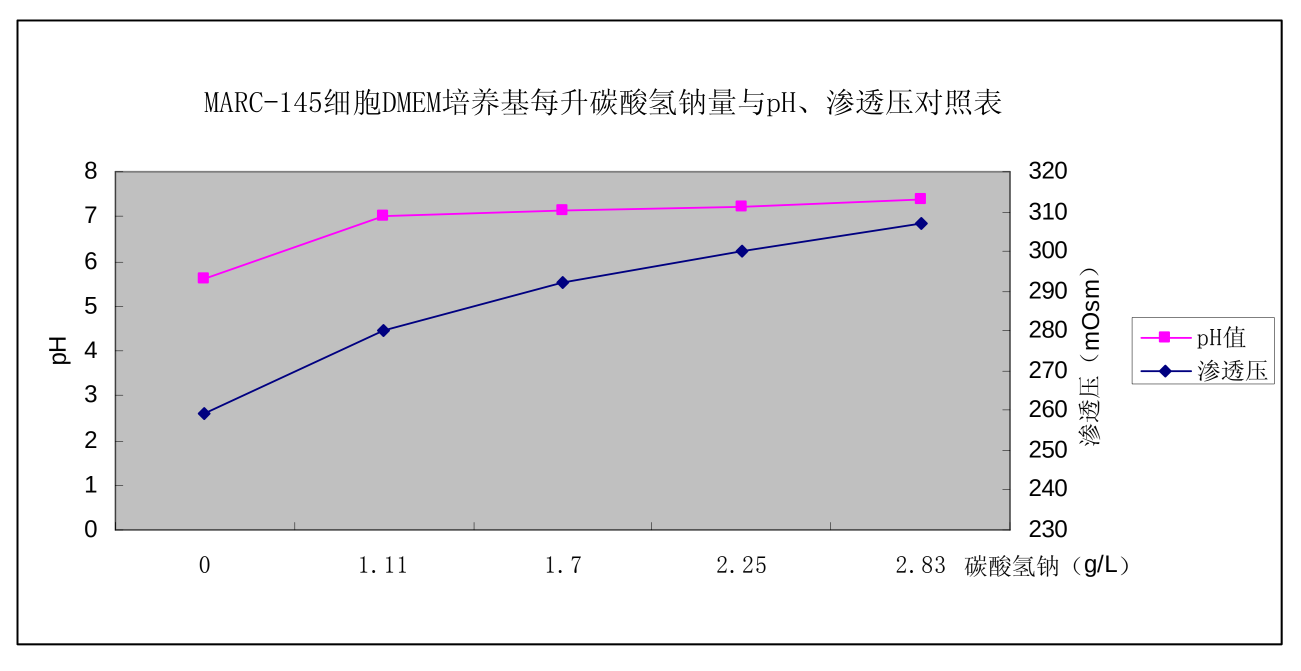
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附录 2. MARC-145 细胞 DMEM 培养基 pH/渗透压对照表

$NaHCO_3$ 加量（g/L）	0	1.11	1.70	2.25	2.83
pH 值	5.60	7.00	7.12	7.20	7.40
渗透压	259	280	292	300	307



附录 3. 参考文献

牛血清在细胞培养中的作用及产生的常见问题

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现代生物技术包括基因工程、细胞工程、酶工程和发酵工程，这些技术的发展几乎都与细胞培养有密切的关系，特别是在生物医药领域，细胞培养更具有特殊的作用。比如基因工程药物或疫苗在研究生产过程中很多都是通过细胞培养来实现的，基因工程乙肝疫苗大多是以CHO细胞作为载体；基因工程抗体药物的制备也离不开细胞培养。细胞工程领域更离不开细胞培养技术，杂交瘤单克隆抗体的研究制备完全是通过细胞培养来实现的，发酵工程和酶工程有的也与细胞培养密切相关。总之，细胞培养在整个生物技术产业的发展中起到了很关键的核心作用。细胞培养的发展，培养基的质量是关键，而培养基的构成中动物血清对细胞的生长繁殖起着重要作用。在动物血清中牛血清的应用又是最广泛的，所以牛血清的质量好坏直接影响生物制品的质量和安全性。

一、牛血清的主要成分

牛血清是一种成分复杂的混合物，其大部分成分已为人所知，但还有一部分仍不清楚，而且血清的组成及含量通常随供血动物的性别、年龄、生理条件和营养条件不同而存在差异。牛血清主要成分有以下几类：

1、蛋白质类 包括白蛋白、球蛋白、α-巨球蛋白（抑制胰蛋白酶的作用）、胎球蛋白（促进细胞附着）、转铁蛋白（能结合铁离子，减少其毒性和被细胞利用）、纤维粘连素（促进细胞附着生长）等；

2、多肽类 主要是一些促进细胞生长和分裂的生长因子，最主要的生长因子是血小板生长因子，还有成纤维细胞生长因子、表皮细胞生长因子、神经细胞生长因子等，虽然在血清中含量很少，但对细胞的生长和分裂也起着重要作用；

3、激素类 激素对细胞的作用是多方面的。包括：

胰岛素：促进细胞摄取葡萄糖和氨基酸，与促细胞分裂有关；

类胰岛素生长因子：能与细胞表达的胰岛素受体结合，从而有胰岛素同样的作用；

促生长激素：促进细胞增殖的效应；

氢化可的松：血清中含有微量该成分，它可能具有促细胞贴附和增殖作用。但有人证明，血清中 的氢化可的松，在细胞密度较高时可能有抑制细胞生长和诱导细胞发生分化的作用。

4、其他成分 如氨基酸、葡萄糖、微量元素等。在合成培养基中这些成分的作用并不明显，与蛋白相结合的微量元素对细胞生长起促进作用。

二、牛血清在细胞培养基中的主要功能

牛血清是细胞培养中用量最大的天然培养基，含有丰富的细胞生长必须的营养成份，具有极为重要的功能。

1、提供能促使细胞指数生长的激素、基础培养基中没有或含量微小的营养物，以及某些低分子营养物质；

2、提供结合蛋白，识别维生素、脂类、金属离子，并与有毒金属和热源物质结合，起解毒作用；

3、是细胞贴壁、铺展生长所需因子的来源；

4、起酸碱度缓冲液作用；

5、提供蛋白酶抑制剂，细胞传代时使剩余胰蛋白酶失活，保护细胞不受损伤。

三、牛血清的分类

根据牛出生时间和血清采制分离方法分为：

1、胎牛血清：八月龄胎牛心脏穿刺取血；

2、新生牛血清：出生 12～24 小时新生牛静脉采血；

高级新生牛血清：采血后静置自然析出的血清；

标准新生牛血清：经离心分离的血清；

普通新生牛血清：融血或微融血的血清；

3、小牛血清：出生三月龄小牛动脉采血分离血清；

四、牛血清的质量要求

（一）WHO公布的《用动物细胞体外培养生产生物制品规程》中的要求

1. 牛血清必须来自有文件证明无牛海绵状脑病的牛群或国家，并应具备适当的监测系统。

2.有些国家还要求牛血清来自没有用过反刍动物蛋白饲料喂养的牛群。

3.要能证明所用牛血清中不含对所生产疫苗病毒的抑制物。

4.血清要通过滤膜过滤除菌，保证无菌。

5.无细菌、霉菌、支原体和病毒的污染，有些国家要求无大肠杆菌噬菌体污染。

6.要求血清对细胞有良好的支持繁殖作用。

（二）我国在对牛血清的质量标准最早在 2000 年版《中国生物制品主要原辅材料质控标准》中提出。包括物理性状、总蛋白，血红蛋白、细菌、真菌、支原体、牛病毒、大肠杆菌噬菌体、细菌内毒素，支持细胞增殖检查。2005 版药典颁布之后，小牛血清质量标准被纳入《中国药典》2005 年版 第三部 生物制品分册。

（三）美国对牛血清的质量要求：

Gibco 和Hyclone等美国主要血清供应商的牛血清都已进入到我国市场，他们对血清质量标准有严格要求，从血清来源到产品质量都有明确规定。

1、血清来源 ：可从世界各地收集胎牛血清，但必须符合其质量标准和美国农业部进口要求。

2、血清的收集：必须符合工业生产的标准，低温采集，一次分离，分离后立即混合冻存。

3、血清的制备：超低IgG胎牛血清、透析血清、γ-射线辐照；

4、除菌过滤：0.22μm和 0.1μm滤膜过滤；

5、成品分装：根据需要分装成不同规格。

五、牛血清质量检定指标

1、化学检定：包括渗透压（240～340）、pH（7.0～8.0）、总蛋白含量（3.5～5.0％）、血红蛋白 （≤0.02％）

2、 微生物检查：细菌和真菌——直接培养法、噬菌体——噬斑法和增殖法 支原体——培养法和DNA染色法 、牛病毒——细胞培养法。要求均为阴性

3、内毒素检测——凝胶法和动态浊度法 要求内毒素含量≤10EU/ml

4、促细胞生长测定（SP2/0-Ag14 标准规定）

1）最大增殖浓度≥1.0×10^{6}个/ml、倍增时间≤20 小时

克隆率＝（阳性孔平均数/培养孔总数）×100％

2）贴壁效率（ VERO细胞、二倍体细胞等）

10％血清——50 或 100 个细胞/孔

贴壁效率（％）＝（每孔克隆平均数/每孔存活的培养细胞平均数）×100

相对贴壁效率＝被测血清贴壁效率/参考血清贴壁效

以上项目检定结果应符合 2005 版《中国药典》第三部的相关规定。血清厂家应在此基础上结合疫苗病毒生产工艺建立企业内控检定项目并建立相应的检定方法，保证疫苗病毒生产的稳定性，提高疫苗制品的质量。

六、牛血清的使用所产生的常见问题

（一）由于血清营养丰富，贮存条件特殊，因此在贮存和使用过程中容易产生以下问题：

1、改变培养液的pH值

牛血清的pH理论值为 7.0～8.0，培养基在加入规定量的碳酸氢钠后（即达到细胞生长所需要的渗透压），再加入 10％的牛血清，一般会使培养液的pH值发生改变。因此在使用过程中应注意pH的变化，如有必要可用 1mol/L NaOH或 1mol/L HCl调pH到所需值。

2、由于血清的营养成分丰富，非常适合细菌、真菌和支原体等生长。国产血清大部分采用手工分装，因此容易把微生物带入而使得血清被污染。使用前如果没有及时发现，将会把污染物带入正在培养的细胞中，而使得细胞不能正常生长。

3、血清在贮存解冻过程中会产生沉淀，这是由于血清中含有大量脂蛋白、纤维蛋白及多肽类物质，在-15℃冷冻保存解冻后就会自然形成沉淀，随着保存时间的延长，沉淀量会增加。因此在融化血清时一般

应先在 4℃放置使之解冻，然后放在室温使血清完全融化，以避免沉淀的增加。添加血清时应避免把沉淀加入培养液，否则由于沉淀的存在会使得所培养的细胞产生大量黑点，或者细胞表面将会覆盖一层油状物质，影响细胞的正常生长。为减少血清的损失，可以把有沉淀的血清收集起来进行离心处理，将上清液加入培养液使用（一般不建议采用过滤的方法除去沉淀，因为沉淀会堵塞滤膜而无法过滤）。

（二）大规模细胞培养中牛血清对细胞及病毒滴度的影响

血清是一种成分复杂的混合物，不同批次血清对细胞生长的促进作用不同。大规模细胞培养中由血清引起的细胞生长状态不佳及病毒滴度的影响表现在以下几个方面：

1、细胞生长速度缓慢，长满单层时间长；从同一细胞瓶中分出两瓶细胞，用同一种培养液，分别加入 10％的对照血清和待检血清，在相同条件下培养 72 小时，会出现对照血清长满单层，而待检血清大约只有 60～70％，这种血清就不适合用来大规模培养细胞。

2、细胞传代后形态不正常，细胞状态发生变化 ；由于血清的质量问题，使原本状态正常的细胞经传代后形态会变差；比如：细胞瓶里会出现一些蠕动的小黑点（并非污染），或者细胞表面形成一层油状覆盖物；细胞的轮廓变得模糊，细胞之间的间隙被血清中的蛋白颗粒填充，从而影响细胞向四周扩展生长。

3、细胞传几代后就出现脱壁现象，不能连续传代 ；质量较差的血清缺乏某种细胞的贴壁因子，会使细胞在传代过程中逐渐丧失贴壁的能力。细胞会从正常的贴壁状态，边缘开始萎缩，上翘。观察到的现象就是细胞表面不光滑，晃动细胞瓶会看到细胞表面有絮状物随培养液波动。随着传代次数增加，细胞萎缩的情况越来越严重，直到最终完全脱壁而死亡。

4、细胞长的很好，致密的单层，状态也很正常，但是接毒后细胞基本不发生病变，做滴度检测几乎测不出抗原滴度。这种情况也许是因为血清中含有与接种的病毒有同源的特异性抗体。比如血清中经常会存在乙脑抗体，牛腹泻病毒抗体等，如果存在这些抗体，就会把接进去的病毒给中和掉。如果抗体滴度很高，病毒会被抗体完全中和，就没有病毒颗粒在细胞中增殖，所以会检测不到病毒滴度。这种情况给疫苗企业造成的损失是相当大的，不产毒等于前面所做的那么多工作全部白费，浪费人力、物力，尤其是时间上的浪费，从培养细胞开始到接病毒，到收液至少需要一两个月，一旦出现不产毒的情况，那么这一两月时间就白白浪费掉了，这也是牛血清给疫苗企业造成的风险之一。

5、细胞生长的状态一般，产毒少，毒价低。这种情况比较常见，细胞是病毒的载体，由于血清质量不好，导致细胞生长状态不好，病毒就无法进行正常的复制。并非所有病毒都不能复制，所以就造成疫苗的效价不够，可能造成不合格产品。

（三）牛血清给生物制品带来的问题

对于使用传统培养基培养的大多数细胞来说，添加牛血清是必不可少的。但是牛血清的使用也给细胞培养和生物制品的生产带来很多问题,并成为生物制品生产水平达到最优化和实现经济性目标的一大障碍。

1、批间差异　血清是一种成分复杂而且不确定的混合物，不同来源的血清成分存在很大的差异，因此支持细胞生长的效果也有较大差别。而每批血清的批量是有限的，所以换血清批号时要预先做大量的筛选实验，以确定对细胞生长影响最小的血清。不但增加工作量,而且如果因为一时筛选不出合适的血清还会导致生产停滞。

2、动物来源成分　因为来源于动物,所以有可能携带传染源,包括病毒和有毒物质,任何一种传染源都有可

能对正在生长的细胞系或者制品带来风险；不同来源的血清存在某些特异性病毒抗体（比如：乙脑抗体、牛腹泻性病毒抗体等），直接影响接毒后病毒的复制，可能导致不产毒或毒价偏低，从而给疫苗生产造成巨大损失。

3、提高生产成本 目前市场销售的血清平均价格约 0.25 元/ml，按 10％添加量计算，1L培养液中血清所占的成本是 25 元，培养基及其他添加物的成本约为 6 元，每升培养液的成本中血清约占 80％，由此可见血清在疫苗生产中占据了大量的生产成本。

4、行业竞争导致质量难以提高 由于国内牛血清生产厂家增多，而用户的数量有限。为了获得客户资源，很多血清厂家都在进行低价销售，行业内形成一种恶性的价格竞争。低价格带来的结果就是低质量的血清。目前很多血清厂家都处于维持生存的状态，因此难以投入大量资金和人力来提高血清的质量。

5、血清蛋白引起的疫苗纯化问题

由于血清含有大量的不同分子量的未知蛋白和多肽类物质，势必会增加提取,分离,纯化的步骤,增加了下游纯化处理的难度,因此在疫苗纯化阶段给纯化工作带来很大的困难；疫苗产品中牛血清白蛋白残留量要求不高于 50ng/剂，为达到纯化的目的，纯化工艺不得不添加新的仪器设备；在除去牛血清白蛋白残留的同时，也会损失大量的有效目的产物，从而降低了疫苗的产率；牛血清中的某些蛋白与目的产物的分子量接近，在纯化过程中不能完全被去除，由于血清残留而导致的生物制品不合格,或者因为杂蛋白含量大造成严重不良反应的情况时有出现.因此也会严重影响疫苗产品的质量。

6、相关法规对牛血清使用的规定

为了使与传染源相关的风险降到最低，国际上存在多个国家之间限制进出口生物材料的国际法规；美国、欧盟和日本等发达国家和地区计划在 2010 年全面停止血清在生物制药中的应用；FDA目前已不受理利用血清进行细胞培养的新药申报；FDA与美国农业部已经严密控制胎牛血清在细胞培养中的使用，疯牛病和口蹄疫的流行导致对牛血清控制使用更加严密；2003 年 4 月，Vera Baumans和Jan van der Valk组织召开题为“改进体外培养技术方法，替换胎牛血清”的会议，讨论FBS的使用问题，在全球范围内号召减少FBS的使用；因此，预计政府部门对胎牛血清加工或使用的限制将会更加严厉。

药品管理法对生物制品的质量提出严格要求，尤其是我国加入WTO之后，在制品的质量上要求更严格。产品的市场竞争应主要靠质量竞争，所以对制品的主要原材料的质量要求也会越来越严格。牛血清作为生物制品生产所需的一种原材料，其质量的好坏直接关系到制品的质量和安全性，由牛血清给生物制品带来的问题也日益凸现。细胞大规模培养及减少动物来源成分在生物制品中的使用已成为今后生物制药发展的趋势，为提高制品的质量和安全性，生物制品生产过程应尽量降低血清的使用量或寻找替代血清及动物组分的原材料。

Porcine reproductive and respiratory syndrome virus (PRRSV) infection spreads by cell-to-cell transfer in cultured MARC-145 cells, is dependent on an intact cytoskeleton, and is suppressed by drug-targeting of cell permissiveness to virus infection

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Abstract

Background: Porcine reproductive and respiratory syndrome virus (PRRSV) is the etiologic agent of PRRS, causing widespread chronic infections which are largely uncontrolled by currently available vaccines or other antiviral measures. Cultured monkey kidney (MARC-145) cells provide an important tool for the study of PRRSV replication. For the present study, flow cytometric and fluorescence antibody (FA) analyses of PRRSV infection of cultured MARC-145 cells were carried out in experiments designed to clarify viral dynamics and the mechanism of viral spread. The roles of viral permissiveness and the cytoskeleton in PRRSV infection and transmission were examined in conjunction with antiviral and cytotoxic drugs.

Results: Flow cytometric and FA analyses of PRRSV antigen expression revealed distinct primary and secondary phases of MARC-145 cell infection. PRRSV antigen was randomly expressed in a few percent of cells during the primary phase of infection (up to about 20–22 h p.i.), but the logarithmic infection phase (days 2–3 p.i.), was characterized by secondary spread to clusters of infected cells. The formation of secondary clusters of PRRSV-infected cells preceded the development of CPE in MARC-145 cells, and both primary and secondary PRRSV infection were inhibited by colchicine and cytochalasin D, demonstrating a critical role of the cytoskeleton in viral permissiveness as well as cell-to-cell transmission from a subpopulation of cells permissive for free virus to secondary targets. Cellular expression of actin also appeared to correlate with PRRSV resistance, suggesting a second role of the actin cytoskeleton as a potential barrier to cell-to-cell transmission. PRRSV infection and cell-to-cell transmission were efficiently suppressed by interferon-γ (IFN-γ), as well as the more-potent experimental antiviral agent AK-2.

Conclusion: The results demonstrate two distinct mechanisms of PRRSV infection: primary infection of a relatively small subpopulation of innately PRRSV-permissive cells, and secondary cell-to-cell transmission to contiguous cells which appear nonpermissive to free virus. The results also indicate that an intact cytoskeleton is critical for PRRSV infection, and that viral permissiveness is a highly efficient drug target to control PRRSV infection. The data from this experimental system have important implications for the mechanisms of PRRSV

persistence and pathology, as well as for a better understanding of arterivirus regulation.

Background

Porcine reproductive and respiratory syndrome virus (PRRSV) is an arterivirus which is the etiologic agent of PRRS, a disease of epidemic proportions in swine [1-3].

PRRSV is macrophage-tropic *in vivo*, where it establishes a chronic infection, and the virus replicates in primary pig macrophages *in vitro* [4-6]. PRRSV infection has been extensively studied in MARC-145 cells, a PRRSV-permissive monkey kidney cell line [7,8]. Previous studies have established that PRRSV replication in cultured MARC-145 cells follows a complex time-course, with PRRSV antigens becoming detectable by immunofluorescence analysis between about 10–20 h p.i., and emergence of foci of damage (cytopathic effect; CPE) usually over the next 3–4days [7,8].

The fate of PRRSV-infected MARC-145 cell cultures may include death of some cells by modified apoptosis [9] or necrosis [10], as well as establishment of chronic PRRSV infection (Cafruny & Rowland, unpublished). Thus, clarifying the behavior of PRRSV in MARC-145 cells is significant to progress in developing anti-viral strategies. Previous studies have suggested that initial defenses against PRRSV are comprised of innate lung and alveolar macrophage responses [6]; subsequently, both Th1 and Th2 responses are induced in the respiratory tracts of PRRSV-infected pigs [11]. PRRSV infection of pigs is associated with activation of several cytokines including interferon-γ [IFN-γ; [12,13]], which has PRRSV-inhibitory activity *in vitro* [14]. However, the IFN-γ response to PRRSV may be inhibited or delayed by some unknown factors during PRRSV infection or vaccination [15,16],and ultimately a poorly-neutralizing Th2-dependent response seems to result in many pigs. Combined, the characteristics of these host responses may facilitate viral persistence [15,16].

The interaction of PRRSV with host cytokines is not well understood, but this area of study is a potential key to understanding host mechanisms during infection. Cytokines have not yet been exploited to control PRRSV infection *in vivo*, but their potential to regulate PRRSV infection in certain experimental systems provides a rationale for PRRSV discovery research, and anti-PRRSV agents may be important tools for future drug development.

The viral dynamics of another arterivirus, lactate dehydrogenase-elevating virus (LDV), are dominated by regulation of the LDV-permissive state; only a small fraction of mouse macrophages are susceptible to LDV infection,leading to an avirulent chronic infection in most mice which is maintained through development of newly-permissive cells [17]. Viral permissiveness is a logical but poorly exploited target for antiviral drugs [18-20], and the present study utilized two antiviral agents which target permissiveness (IFN-γ and an experimental antiviral known as AK-2), as well as cytoskeleton disruptors, to probe checkpoints in PRRSV replication.

Initially, our goal was to better characterize the dynamics of PRRSV replication in MARC-145 cells. Using flow cytometry and fluorescence microscopy, we demonstrated logarithmic growth of PRRSV in MARC-145 cells, culminating over a period of 3–4 days in the death of most cells. Secondary spread of PRRSV infection was observed to be via cell-to-cell transmission, as demonstrated by emergence of clusters of PRRSV-infected cells in

confluent monolayers of MARC-145 cells, which preceded PRRSV induced CPE, were inhibited by colchicine and cytochalasinD, and correlated with reduced actin expression. PRRSV replication was sensitive to IFN-γ as well as AK-2, which was a relatively more potent PRRSV inhibitor and capable of suppressing both primary and secondary PRRSV infection. The results of this study demonstrate cell-to-cell spread of PRRSV in cultured MARC-145 cells, the dependence of PRRSV infection and transmission on an intact cytoskeleton, and highlight the role of the PRRSV-permissive state as a critical drug target, with important implications for future therapeutic and preventive strategies.

Results

PRRSV replication dynamics in MARC-145 cells

When PRRSV replication was assessed in MARC-145 cells at 20–22 h p.i., only a small proportion of cells expressed PRRSV antigen, as determined by FA (<5% in > 10 manual experiments counting fluorescent-positive cells under the microscope; Figure 1 by flow cytometry). However, between 42–72 h p.i. the percent of PRRSV-positive cells increased rapidly as determined by flow cytometric analyses, up to a maximum of about 95% by 96 h p.i. (Figures1 &2). Low permissiveness of MARC-145 cells to primary (less than about 22 h p.i.) PRRSV infection was not due toinsufficient M.O.I., since inoculation of cultures with about 100-times the standard dose resulted in a maximum 5.5% incidence of PRRSV-positive cells at 22 h p.i. by flow cytometry (data not shown). Propidium iodide staining followed by flow cytometry showed that PRRSV inoculated cells underwent major changes in cell cycle parameters (e.g. drop in G1 and G2; increased debris and aggregates) between 72–96 h p.i., consistent with cell damage and the spread of microscopic foci (CPE) typically observed throughout the cultures (data not shown).

FA analyses by confocal fluorescence microscopy, of dozens of independent PRRSV-infected confluent cultures, revealed that the primary (< 22 h p.i.) phase of PRRSV infection was characterized by random targeting of PRRSV-permissive single cells (Figure 3A; see also Figure 5B for another example), representing only a few percent of the total cells, and consistent with the quantitative analyses by flow cytometry.

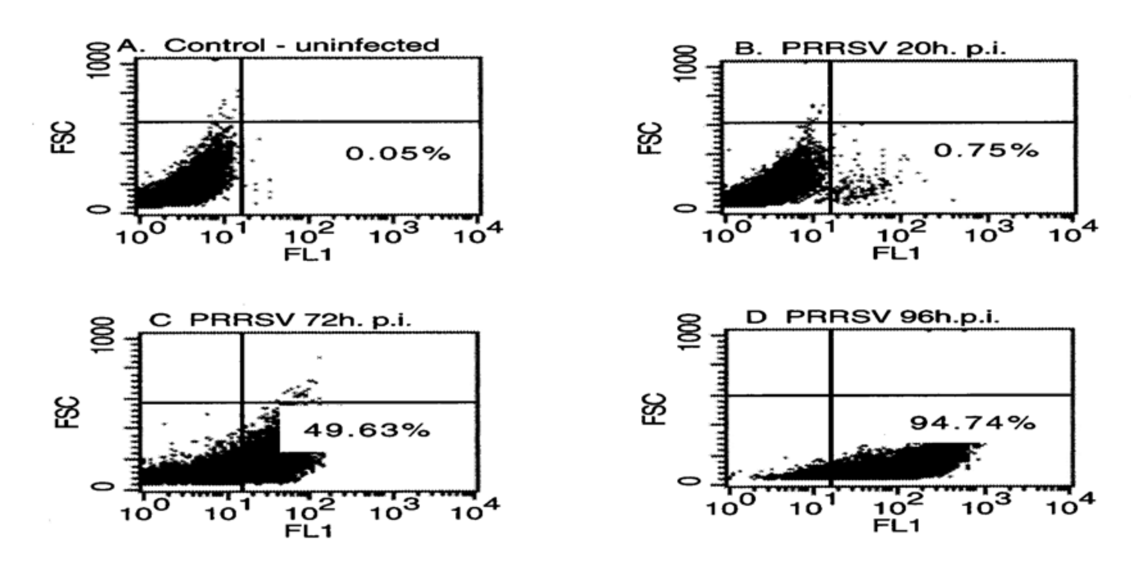


Figure 1 PRRSV replication dynamics in MARC-145 cells by flow cytometry.

A. Control (uninfected) cells; B. 20 h p.i. with PRRSV; C. 72 h p.i. with PRRSV; D. 96 h p.i. with PRRSV. The percentage of PRRSV antigen-positive cells is shown in the lower right quadrant for each graph.

Formation of PRRSV-infected cell clusters (secondary infection)

During the logarithmic phase of viral replication (e.g. 42–48 h p.i.), infection was present mainly in clusters containing multiple PRRSV-positive MARC-145 cells, routinely observed against a PRRSV-negative background of confluent cells (examples indicated by arrows in Figures 3B,C,D; see also Figures 5C,D and 6B). These clusters were never observed in the primary infection analyses, although occasionally infected cell doublets were seen at 20–22 h p.i.. Based on >10 independent analyses, we estimate that about 90% of secondary PRRSV infection of MARC-145 cells line is characterized by formation of infected cell clusters. Typically, dozens of clusters were seen in each culture, often containing a relatively central, bright-staining cell (e.g. Figure 3B, D), suggesting that cell-to-cell spread originated from a single PRRSV-infected reservoir cell.

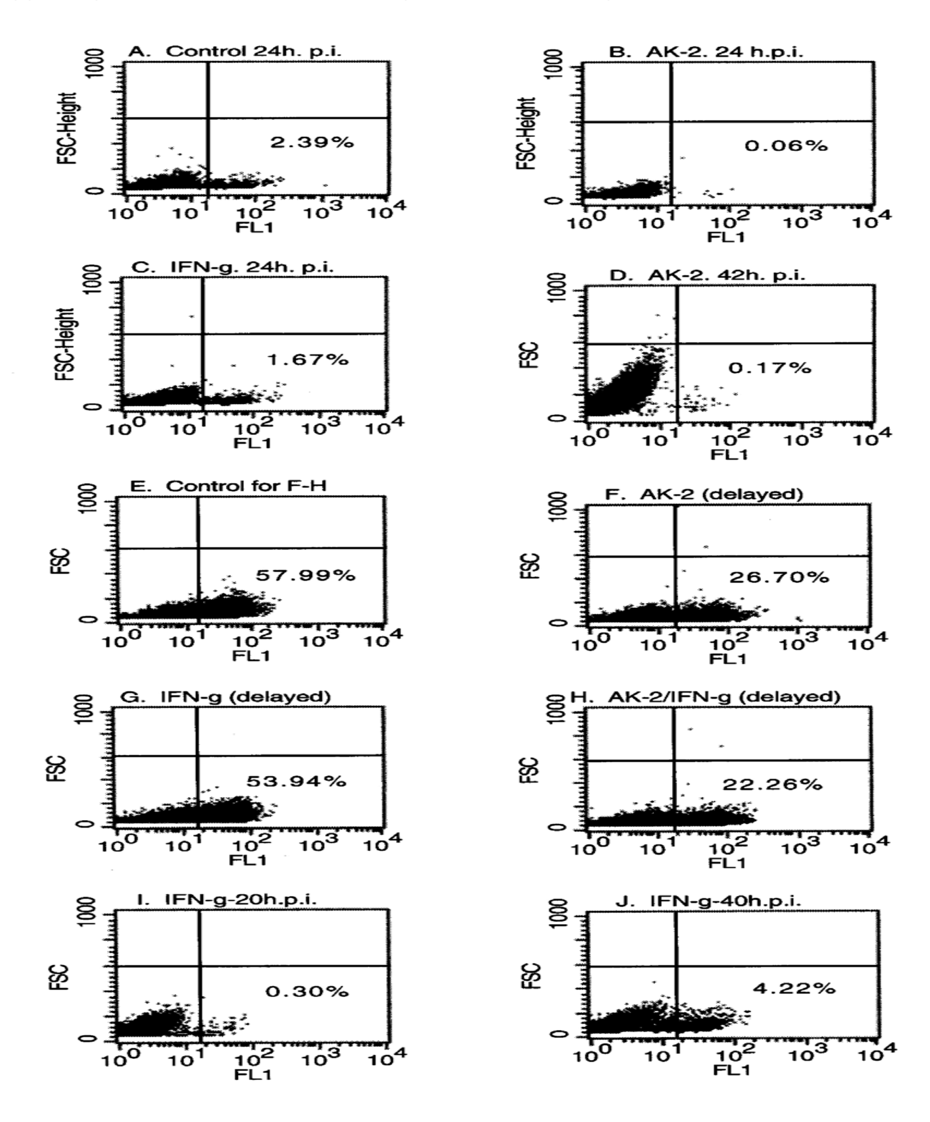


Figure 2 Effects of AK-2 and IFN-γ on PRRSV replication in MARC-145 cells as determined by flow cytometry.

A. Control 24 h p.i. with PRRSV; B. AK-2 pretreatment, 24 h p.i. with PRRSV; C. IFN-γ pretreatment, 24 h p.i. with PRRSV; D. AK-2 pretreatment, 42 h p.i. with PRRSV; E. Control 46 h p.i. with PRRSV; F. AK-2 started at 18 h p.i. (delayed); G. IFN-γ started at 18 h p.i. (delayed); H. AK-2 and IFN-γ in combination, started at 18 h p.i.; I & J. IFN-γ-pretreatment effects on primary (I) and secondary (J) response PRRSV antigen detection. The percentage of PRRSV antigen-positive cells is shown in the lower right quadrant for each graph.

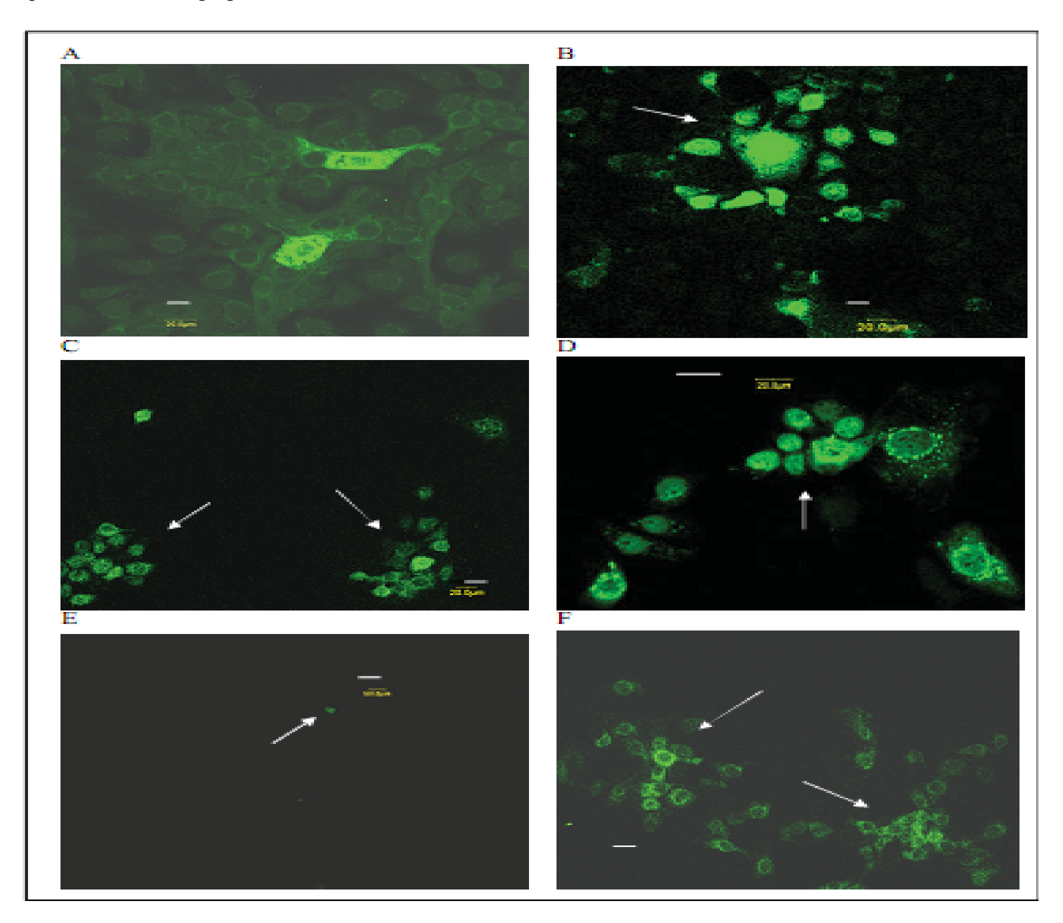


Figure 3 Confocal microscopy of FA-stained MARC-145 cells during PRRSV infection.

A. 24 h p.i. with PRRSV, 20 µm.; B-C. Secondary cluster formation (arrows) 42–46 h p.i. with PRRSV, 20 µm; D. Secondary cluster formation (arrow) 72 h p.i. with PRRSV, 20 µm.; E. AK-2 pretreatment, 42 h p.i.; arrow indicates a single PRRSV-positive cell, 50 µm; F. AK-2 post-treatment, 46 h p.i., arrows indicate several PRRSV-positive clusters, 20 µm. (Length of scale bar is indicated for each panel)

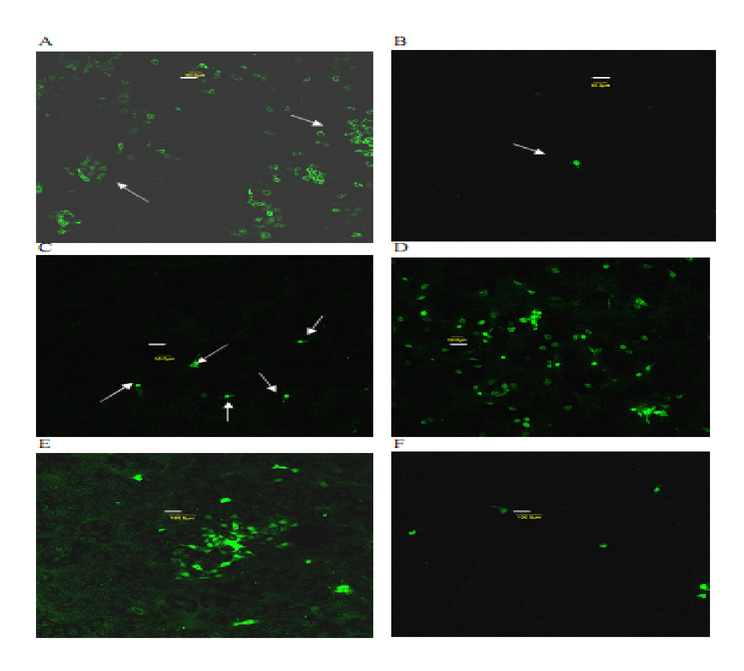


Figure 4 Confocal microscopy of FA-stained MARC-145 cells during PRRSV infection.

A. IFN-γ pretreatment, 46 h p.i., arrows indicate several PRRSV-positive clusters, 50 μm; B.AK-2+ IFN-γ post-treatment, arrow indicates a single PRRSV-posi-tive cell, 46 h p.i., 50 μm;

C. AK-2 pretreatment, 42 h p.i., occasional PRRSV-positive cells (arrows), 50 μm; D. IFN-γ pretreat-ment, 50 μm; E. 45 h p.i. with PRRSV, 100 μm; F. Simultaneous AK-2 treatment and PRRSV infection, 45 h p.i., 100 μm. (Length of scale bar is indicated for each panel)

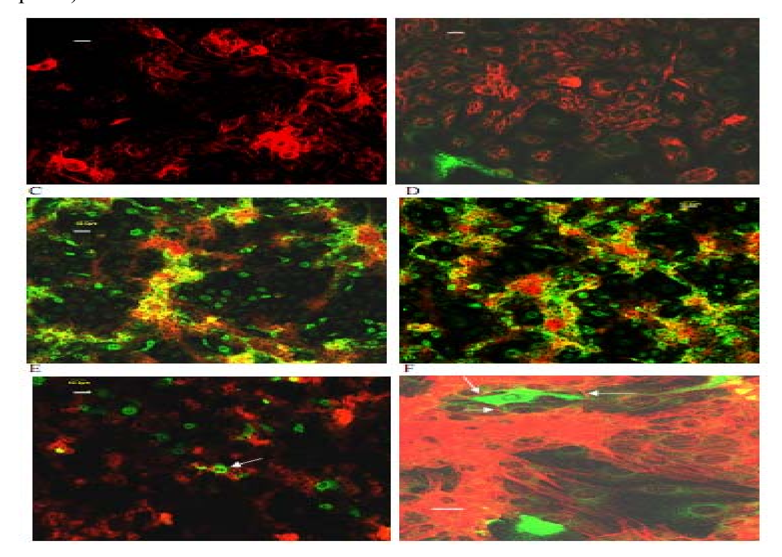


Figure 5 Two-color fluorescence detection of actin (red) and PRRSV antigen (green) in MARC-145 cells.

A. Uninfected control, 20 μm; B.18 h p.i. with PRRSV, 20 μm; C. Control at 42 h p.i. with PRRSV, 50 μm; D. Drug vehicle control at 42 h p.i. with PRRSV, 50 μm. E. Colchicine-treated, 5 μM, 42 h p.i. (arrow indicates PRRSV-positive doublet; see Figure 6, A, C and D for higher magnification), 50 μm; F. AK-2 treated, 250 μm; (Length of scale bar is indicated for each panel)

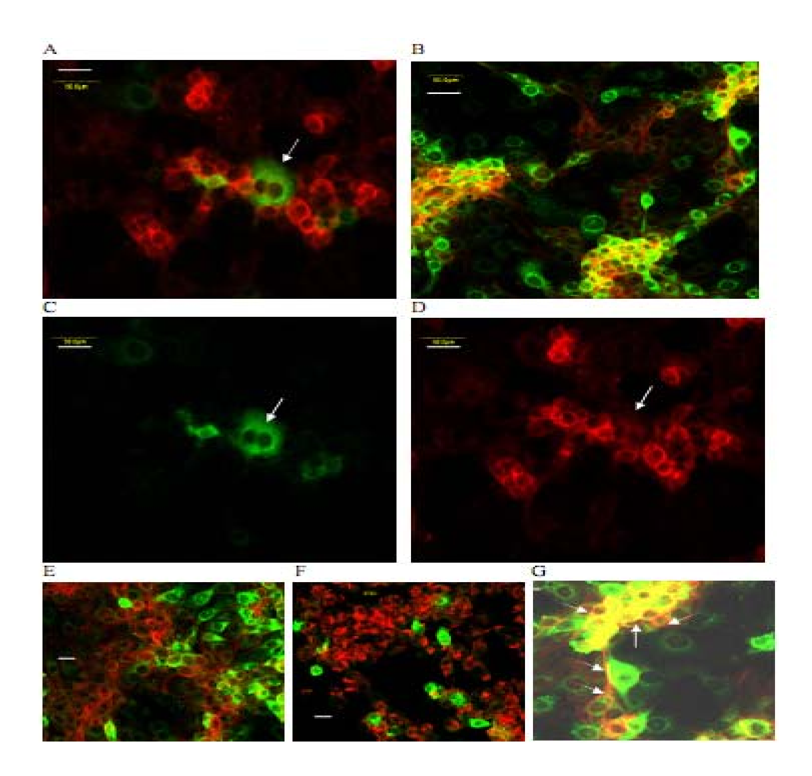


Figure 6 Two-color fluorescence detection of actin (red) and PRRSV antigen (green) in MARC-145 cells.

A. PRRSV-infected cell doublet (arrow) in a colchicine-treated culture; higher magnification, 50 µm; B. Control – higher magnification, 50 µm; C. Arrow indicates PRRSV-infected cell doublet; green-only (compare to red + green in A), 50 µm; D. Arrow indicates position of PRRSV-infected cell doublet, red-only (compare to red + green in A), 50 µm; E. Emerging clusters at 41 h p.i., 20µm; F. Cytochalasin D treatment (1 µM) at 18 h p.i., 20 µm; G. control 42 h p.i. with PRRSV (arrows = actin fibrils; 10 µm). (Length of scale bar is indicated for each panel)

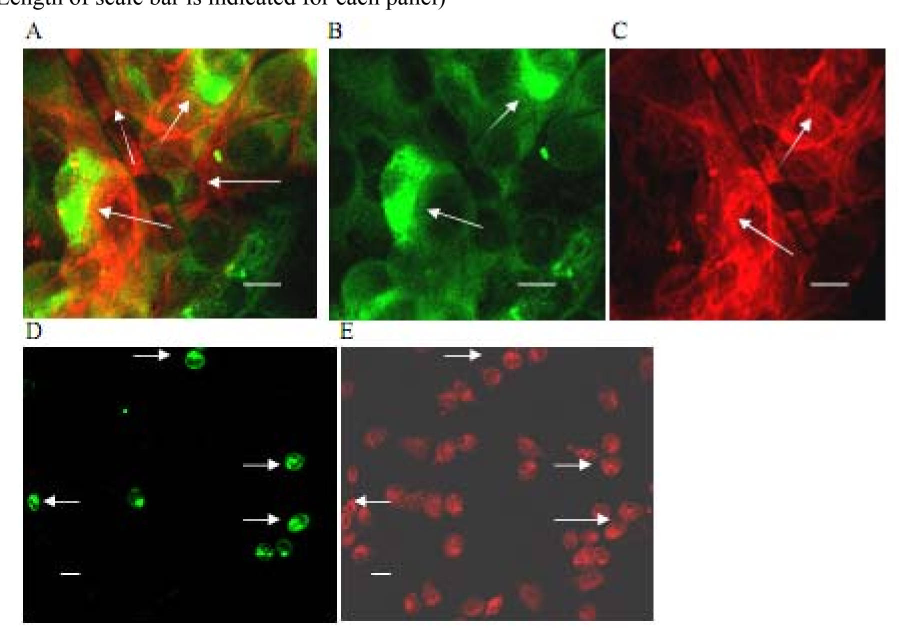


Figure 7.Two-color fluorescence detection of actin (red) and PRRSV antigen (green) in MARC-145 cells.

A-C. Control 41 h p.i.: A = actin (red) + PRRSV Ag (green); B = PRRSV Ag only; C = actin only; arrows = actin fibrils surrounding uninfected cells, 25 µm; D (LDV antigen) & E (actin) expression in LDV-infected primary mouse macrophages; arrows indicate

LDV-infected cells; 10 μm. (Length of scale bar is indicated for each panel).

Absence of cluster formation during primary infection did not appear to be due to insufficient M.O.I. or late-stage development of a soluble cluster-inducer during culture, since using up to about 100-times the standard virus dose (obtained from 45–96 h p.i. cell supernatants) did not induce cluster formation at 20–22 h p.i. in our experimen-tal system (data not shown), and cluster formation was also density-dependent (see below – Figure 8B). Clusters of PRRSV-positive cells were maintained for up to 72 h p.i. as illustrated in Figure 3D, and images at this time were also suggestive of maintenance of central bright-staining cells as reservoirs of virus.

Role of the cytoskeleton in PRRSV infection

Treatment of cells with 10 μM colchicine simultaneously with PRRSV inoculation, resulted in about 75% inhibition of secondary PRRSV infection (e.g. 70% in control vs.18% in colchicine-treated culture; counting >1000 total cells at 46 h p.i.) and data representative of numerous experiments are shown in Figure 5E to illustrate the inhibitory effect of colchicine on formation of PRRSV-infected secondary clusters, which are seen spreading throughout the controls (Figure 5C, D). Similarly, exposure of cells to cytochalasin D under several experimental conditions (1 or 2 μM; administered either simultaneously with virus infection or 2 h pre-infection) inhibited PRRSV-positive cells by up to 87% (illustrated in Figure 8A between 13–22 h p.i.; 2h pre-treatment with 1 μM). These drug effects were confirmed by flow cytometric analyses (about 50–90% inhibition of PRRSV-positive cells; data not shown).

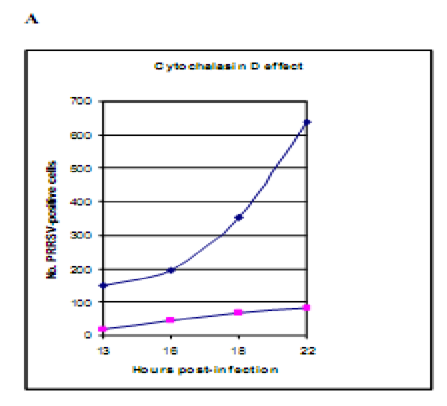
To further evaluate PRRSV transmission *in vitro*, actin expression was imaged using Alexa Fluor 594-phalloidin (red). Similar patterns of actin expression were observed in control uninfected (Figure 5A) and PRRSV-infected (Figure 5B) cells, and all cells expressed some degree of actin when high levels of gain were applied. Cells often displayed fibrillar actin extensions appearing along the outer membrane as well as less-elongated extensions over the body of the cell. Effects on the actin staining pattern were apparent after exposure of MARC-145 cells to the actin disruptor cytochalasin D (Figure 6F), and also to some extent after exposure to the microtubule inhibitor colchicine (Figure 5E) although intact actin fibrils were still present (see also description of Figure 6A, C, D below). Simultaneous determination of actin expression and PRRSV infection demonstrated that relatively high expres-sion of actin filaments correlated with PRRSV resistance, which was a consistent finding in dozens of control- and colchicine-treated experiments. This observation is illustrated in Figure 6A, C, &6D, where the arrows indicate the location of a PRRSV-positive doublet with low actin expression. Figures 5B and 6E also illustrate the negative correlation between actin and PRRSV-antigen expression. While this was not an absolute correlation (some PRRSV-positive cells did express high levels of actin), the trend was clear from >5 independent experiments. Actin fibrils also appeared to partition PRRSV-positive from PRRSV-negative cells and were often observed surrounding PRRSV-negative cells (Figures 6G and 7A–C; and see below). Cytochalasin D added at 18 h p.i. also suppressed secondary PRRSV-positive cluster formation (Figure 6F;41 h p.i.; see emerging clusters in the control for this experiment Figure 6E), demonstrating a role for the cytoskeleton in cell-to-cell transmission. The confluent cultures used in our experiments generally had mean cell-to-cell distances of < 2 μm, and the cells

were usually in contact or too close to measure meaning-ful distances. Due to the wide dynamic range of the fluo-rescence signals, this is not always apparent in the figures, but some figures, such as 3A, 7A &7B, serve to illustrate the background of cultured cells. By seeding plates with different numbers of cells and then infecting these cultures containing different cell densities, it was possible to measure cell-to-cell distances in hundreds of cells per culture from the captured images, and then correlate the mean with the number of PRRSV-positive clusters (Figure8B). The results show that formation of PRRSV-positive secondary clusters was density-dependent, with a direct correlation between the cell-to-cell mean distance and the number of clusters (Figure 8B).

In contrast to the ability of PRRSV to spread via cell-to-cell transmission, LDV did not exhibit this property, nor was there any dissociation of actin expression by primary cultured mouse macrophages from LDV permissiveness (probed during the replication peak, Figure 7D &7E). Also in contrast to the response of PRRSV in MARC-145 cells, treatment of mouse macrophages with the same concen-trations of colchicine had little effect on LDV replication (+17%, -26%, and -21% LDV-positive cells relative to control in three separate experiments), although LDV infection was completely suppressed by cytochalsin D (no LDV-positive cells detected in three separate experiments).

Combined, these data show that after primary acute PRRSV infection of a small subpopulation of PRRSV-per-missive MARC-145 cells, the virus spreads secondarily over the next 2–3 days to surrounding cells by cell-to-cell transmission. These virus mechanisms are both actin- and tubulin-dependent, demonstrating the critical role of the cytoskeleton in the processes of PRRSV infection and spread. In contrast, the related arterivirus LDV displays no cell-to-cell transmission in primary culture mouse macro-phages and primary LDV replication is not tubulin-dependent as revealed by absence of colchicine sensitivity.

A



B

number of clusters

Cell Distances (um)

Figure 8 Time-course of PRRSV infection in cytochalasin D-treated (1 µm pretreatment) and control MARC-145 cells (A), and relation-ship of cell-to-cell distance and formation of secondary PRRSV-infected cell clusters (B).

Suppression of PRRSV infection by targeting viral permissivness with AK-2 and IFN-γ

Pretreatment of MARC-145 cells for about 18 h prior to PRRSV inoculation with AK-2 suppressed primary (Figure 2B) and secondary (Figure 2D) virus infection as assessed by flow cytometry. The pharmacodynamics of AK-2 inhibition of primary PRRSV infection were determined by microscopic FA analyses (Figure 9), demonstrating that the antiviral effect of AK-2 is on the PRRSV-permissive state rather than directly on the virus, since pretreatment was required to fully establish PRRSV resistance. No detectable morphological effects of AK-2 on MARC-145 cells were noted in our studies, and the PI profiles of treated cells were similar to those of control cells demon-strating intact and metabolically viable cells at 22–46 hp.i. (data not shown). AK-2 pretreatment completely inhibited the formation of secondary clusters of PRRSV infection, and only occasionally were single-positive cells observed at 42–46 h p.i. in AK-2-pretreated MARC-145 cells (Figures 3E &4C). However, when added at 20 h p.i., AK-2 only partially inhibited secondary (cluster) PRRSV spread, although there was a shift to single-cell PRRSV infection (Figure 3F). Partial inhibition of secondary PRRSV infection by delayed AK-2 addition was also observed by flow cytometry, since AK-2 added at 18 h p.i. inhibited the 42 h p.i. expression of PRRSV antigen, by about one-half (27% vs. 58% in the drug-vehicle control;Figure 2F). Exposure of MARC-145 cells to AK-2 simulta-neously with PRRSV inoculation suppressed all secondary cluster formation at 45 h p.i. (see control in Figure 4E and AK-2-treated in Figure 4F), confirming that, even without pretreatment, AK-2 can suppress the viral permissiveness of target cells for secondary cell-to-cell transmission, while leaving primary single-cell infection relatively intact due to the time required for induction of PRRSV resistance.

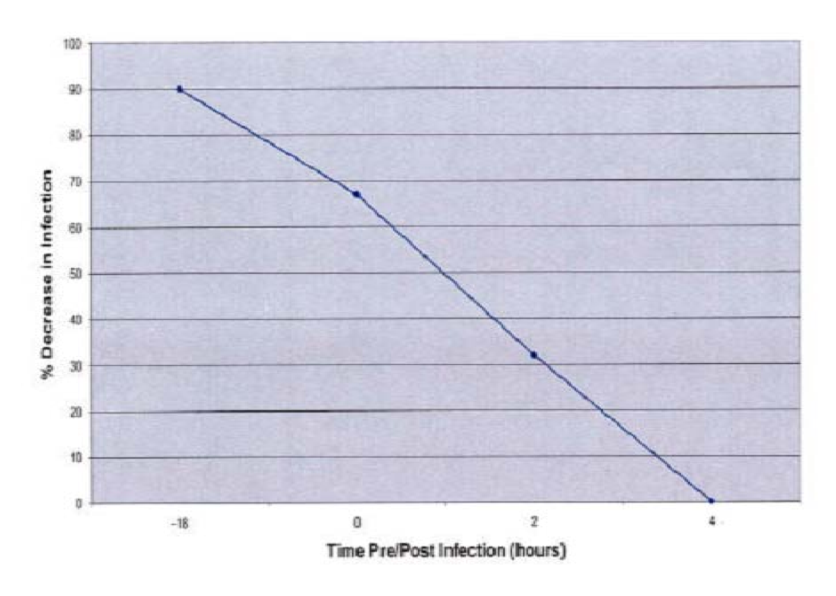


Figure 9 Pharmacodynamics of PRRSV inhibition by AK-2. MARC-145 cells were exposed to AK-2 at various times pre- or post-infection. The % inhibition of PRRSV antigen detection is shown on the y-axis.

A higher-magnification image of AK-2-treated (single-cell infection only) is shown in Figure 5F, which also

illustrates the often-observed appearance of actin fibrils surrounding a PRRSV-positive cell, as if to separate it from other uninfected cells as indicated above for Figure 6G. The data also show the absence of a detectable effect of AK-2 on actin expression.

In other studies, AK-2 pretreatment inhibited PRRSV antigen expression at 20–22 h p.i. by about 90% in primary pig macrophages, in each of two experiments. Similarly, pretreatment of primary mouse macrophages with recombinant murine AK-2 inhibited LDV replication by 86% in a single experiment. Thus, the anti-arterivirus effects of AK-2 are expressed over a broad host cell range.

Observations were also made for the anti-PRRSV effect of IFN-γ pretreatment, which was demonstrated in previous studies to inhibit PRRSV replication [14]. PRRSV inhibition by IFN-γ was less effective than that of AK-2 under the same experimental conditions, as seen for example in the primary response to pretreatment measured by flow cytometry (Figure 2C,I); the effect of IFN-γ pretreatment also waned by 40 h p.i. (Figure 2J). FA analyses confirmed the reduced efficacy of IFN-γ relative to AK-2, since IFN-γ pretreatment did not completely inhibit formation of secondary PRRSV antigen-positive clusters (Figure 4A,D) and there were higher numbers of PRRSV antigen-positive cells in IFN-γ-pretreated cultures (data not shown). Consistent with these data, flow cytometry demonstrated that IFN-γ added at 18 h p.i. had a reduced effect on secondary PRRSV infection (Figure 2G), relative to AK-2 (Figure 2F) or the combination of AK2 and IFN-γ (Figure 2H); the inhibitory effect of post-treatment with both drugs is also shown in Figure 2H, although little if any synergy was observed. Thus, while IFN-γ mediated significant inhibition of PRRSV infection, AK-2 appears to be a more potent anti-PRRSV agent.

Discussion

The results of this study show that PRRSV replication in an experimental MARC-145 cell system is composed of two discrete phases: primary infection of a relatively small subpopulation of PRRSV-permissive cells during about the first 22 h p.i, followed by secondary cell-to-cell spread over the next several days to contiguous cells, resulting in formation of infected cell clusters and ultimately cell death/CPE by days 3–4 p.i. Flow cytometry of PRRSV infection of pig macrophages has previously been reported [32], but for the present study we developed a flow technique to quantitatively measure PRRSV antigen expression in MARC-145 cells, a standard cell line for the study of PRRSV infection. Combined with FA analyses by microscopy, this methodology provides evidence that per-missiveness to PRRSV infection is dependent on the mechanism of virus presentation, since the majority of cells appear non-permissive to free virus, but become readily infected by exposure to productively-infected cells.

These dynamics of PRRSV infection of MARC-145 cells stand in stark contrast to those of the related and relatively benign arterivirus LDV, since primary LDV infection of cultured mouse macrophages peaks at about 8 h p.i. *in vitro*, and soon thereafter *in vivo*, but there is little or no secondary virus replication after these events [17]. A likely explanation for this difference is the absence of cell-to-cell (cluster) spread of LDV. The ability of PRRSV to spread secondarily by cell-to-cell transmission may overcome an early block to virus permissiveness,

and while it is not yet known whether this mechanism occurs *in vivo*, it could potentially help PRRSV resist antibody defenses and maintain persistence.

Our studies show that PRRSV transmission to infected cell clusters is dependent upon cytoskeletal function, since the microtubule inhibitor colchine [26-28] as well as the actin inhibitor cytochalasin D [26, 29-31] suppressed secondary virus spread. Consistent with this conclusion, a number of other viruses are dependent on the host cytoskeleton for entry, transport, and/or egress [32-34], and actin polym-erization may enhance cell-to-cell virus spread [35]. For example, the actin cytoskeleton is a critical factor for assembly and/or budding of HIV-1 [36], West Nile virus [37], respiratory syncytial virus [31], fowlpox virus [38], and equine infectious anemia virus [39]. Interestingly, we observed that the pattern of actin expression correlated with PRRSV resistance, since there was often a distinct appearance of actin filaments surrounding PRRSV-negative cells, and a general (but not absolute) negative corre-lation between viral antigen detection and the level of actin expression. This finding appears to suggest that actin provides a protective barrier to cell-to-cell transmission, and that the actin cytoskeleton may have a dual role in PRRSV infection. This seemingly paradoxical observation may be analogous to that reported for transport of secretory granules, which is both limited and mediated by the actin cortex [40]. Furthermore, the actin cytoskeleton is a potential barrier to exocytosis [41, 42], and the cortical actin network may provide a cellular barrier to SFV [43] and HIV [29]. PRRSV infection might potentially inhibit the cytoskeleton [44-46], to further promote virus spread in culture. In contrast to the finding with PRRSV, there was no difference between actin expression in LDV-per-missive and LDV-non-permissive mouse macrophages.Additional studies of the role of the cytoskeleton should be of interest to PRRSV pathogenesis and the biology of arteriviruses.

Primary infection was dependent on an intact cytoskeleton, and nascent cluster initiation during this time frame was signified by the occasional appearance of infected cell doublets. Formation of secondary PRRSV-infected cell clusters was a function of time p.i. and the cell-to-cell distance, and is thus a physical property of the in vitro system, potentially analogous to tissue sites *in vivo*. The viral dynamics from our studies are consistent with previous observations demonstrating infection of a small percentage of cells by day 1 p.i., which increases markedly over the next few days, culminating in peak supernant virus titers at about 72–96 h p.i. [9]. The data also suggest that 1$TCID_{50}$ contains multiple virions since the number of cells acutely infected can exceed the $TCID_{50}$ dose and optimal infection is achieved at low M.O.I. (calculated by $TCID_{50}$) as previously reported (9, 14). Future studies to clarify the relationship of M.O.I and $TCID_{50}$ might help to determine what special characteristics facilitate primary permissiveness to free virus, which could include ability to bind one or more virions as well as biochemical factors regulating virus replication.

Our data show that the logarithmic increase in the percentage of PRRSV infected cells over about 2–4 days p.i. is due to secondary cell-to-cell virus spread, from innately- permissive (reservoir) cells to surrounding uninfected cells. The foci of infection typically observed microscopically, in cultures of PRRSV-infected cells which begin to degenerate by 3–4 days p.i., are thus the outcome of secondary cluster infection and direct virus infection. These data reinforce that secondary spread to clusters in MARC-145 cells provides an important direction for future

studies of PRRSV mechanisms, since cell-to-cell virus transmission [47] might help to explain the resistance of PRRSV to antibody-mediated control as well as PRRS pathology. In a recent study, the replication of PRRSV in transformed pig peripheral blood monocytes was shown to be under genetic control and varied between 23.1–31.4% at 24 hp.i. [48]. Thus, our results suggest the possibility that variations in cell-to-cell PRRSV transmission may underlie differences in PRRSV replication between different cell lines *in vitro*.

The present results show that AK-2 is a potent inhibitor of arterivirus (PRRSV and LDV) replication. This is the first published report of the antiviral effects of AK-2, which suppresses viral permissiveness by activating an antiviral gene program (Wong; unpublished), and which we exploited to supplement our studies of the IFN-γ response. Pretreatment was required for full expression of the drug effects, likely due to a lag phase for activation of the antiviral gene program. Both primary as well as secondary (cluster) PRRSV infection were susceptible to the antiviral actions of AK-2, but required optimal conditions of pre-treatment for induction of the PRRSV-resistant state, and secondary PRRSV infection was controlled independently of primary infection by simultaneous or delayed drug exposure. IFN-γ was relatively less effective under our experimental conditions, but our IFN-γ data reinforce the conclusion that the viral-permissive state is an important drug target in PRRSV infection. This is also the case for LDV-mediated fetal infection [49, 50] and neuropathology [51], since suppression of LDV-permissiveness by IFN-γ reduces these viral phenomena, and the arteriviruses may be good models for the role of permissiveness in antiviral strategies. Despite sensitivity to IFN-α, PRRSV may be a relatively weak inducer of this cytokine [52], facilitating evasion of host defenses. Thus, development of useful drugs which target viral permissiveness could be a superior strategy to inhibit primary or secondary phases of PRRSV infection, particularly if secondary cell-to-cell spread is resistant to a conventional antibody attack, and might also provide a superior toxicity profile, since the induction of PRRSV resistance is fundamentally a physiological process.

Conclusion

PRRSV infection has been shown to spread by cell-to-cell transmission in a stable MARC-145 cell line. Two stages of viral infection have been identified: primary (innate) per-missiveness to free-virus which appears in a relatively small percentage of cells, and secondary permissiveness to cell-to-cell transmission which is highly expressed and culminates in CPE. PRRSV infection of MARC-145 cells requires an intact cytoskeleton, but actin expression may also correlate with cell protection. Drugs such as AK-2 which induce a block in PRRSV permissiveness reveal a potentially important drug target for suppression of primary and secondary PRRSV infection.

Methods

MARC-145 cells

A stable and mycoplasma-free MARC-145 cell line was utilized in these experiments. Cells were cultured in DMEM containing10% fetal bovine serum, and for virus infections the medium was switched to MEM containing 2% horse serum. Cells for virus infections were grown to confluency in either T-25 flasks (seeded with about 5 $\times 10^5$ cells/culture) or 8-well glass slide chambers (seeded with about 10,000 cells/culture; Lab-Tek II; Nalge Nunc

International), and for the cell density studies, serial two-fold dilutions of the cells were used. Cells were inoculated with PRRSV at about 1–2 days after seeding (time to approximate doubling of the population).

Primary pig macrophages

Pig cells were collected from 4–8 week old pigs by lung lavage with PBS [21-23]. Cells were cultured in DMEM containing 10% FBS. After 18–24 h, non-adherent cells were removed by washing. The remaining adherent cells were cultured for an additional 24 h in RPMI containing 2% horse serum, and then inoculated with PRRSV (M.O.I.approximately 0.1 $TCID_{50}$).

PRRSV stocks

PRRSV isolate SD-23983 was passaged on MARC-145 cells, preparing high-titer (~ 10^5 $TCID_{50}$ per ml) virus stocks from culture supernatants at 48–96 h p.i. PRRSV stocks were sequentially filtered through 0.45, 0.22, and 0.10 um filters and confirmed to be mycoplasma-free by testing on PPLO medium. As reported previously [9,14], maximum efficiency of PRRSV infection of MARC-145 cells occurs with low M.O.I as determined by $TCID_{50}$, probably due to the presence of multiple virions per $TCID_{50}$. For the present studies, M.O.I. of about 0.01 $TCID_{50}$ (slide cultures) and 0.001 $TCID_{50}$ (T-flask cultures) were found to result in near-optimal efficiency of infection, and were thus used for our studies unless otherwise noted in Results.

Fluorescence (FA) detection of viral and cellular target molecules

PRRSV replication was detected using FITC-labeled IgG anti-PRRSV nucleocapsid monoclonal antibody (SDOW17; [24]). MARC-145 cells were cultured and inoculated with PRRSV in glass-bottom slide chambers, fixed in 80% acetone, and incubated for 1 h at 37°C with a 1:100 dilution of FITC-conjugated SDOW17 antibody made in PBS containing 5% fetal bovine serum. Then the cells were washed three times with cold PBS prior to examination under a fluorescence microscope, screening about 30–40,000 total cells to obtain the incidence of antigen-positive cells. Confocal fluorescence microscopy was performed using an Olympus BX61 microscope and Fluoview software. Images shown in Figures 3 and 4 display the yellow scale bar captured with the original image, along with a higher-contrast white scale line. For flow cytometry, MARC-145 cells were cultured and PRRSV-inoculated in T-flasks, the cells were suspended in trypsin-versene, pelleted at 1000 rpm, resuspended in DMEM with 2% horse serum, fixed in cold 80% acetone for 10min, washed twice in PBS, and resuspended in 1 ml PBS containing 60 ul of fetal bovine serum. FITC-conjugated SDOW17 antibody was then added to the cells (2.5 ul/ml), incubation was carried out at 37°C for 60 min, the cells were washed with PBS, examined under a fluorescence microscope, and flow cytometry was performed with a FACS Vantage SE (Becton Dickenson) equipped with a 488 Enterprise II coherent laser. Twenty thousand events per sample were analyzed with Cell Quest software.

Cell cycle analyses were also performed on the same samples, by staining with propidium iodide for 20 minutes at room temperature and analyzing the flow cytometric results with ModFit LT 2.0 software. PRRSV replication in primary pig alveolar macrophages [22, 23] was assessed by FA under a fluorescent microscope. Cellular expression of actin was determined by incubating acetone-fixed cells with AlexaFluor 594 phalloidin (Invitrogen) according

to the manufacturer's instructions, with the modification of simultaneous PRRSV detection as above, such that combined labels were applied for 60 min at 37°C, permitting two-color fluorescence detection by confocal microscopy. Data shown are representative of at least 2 replicate experiments for each type of experiment described in the Results.

LDV infection of primary mouse macrophages

Peritoneal macrophages were collected from outbred ICR mice, seeded onto glass coverslips, and inoculated with a standard dose of LDV-P as described previously [25]. LDV replication was assessed in cells fixed in acetone at 8 h p.i by IFA assay as described previously [25].

Drug treatments

Purified recombinant human interferon-γ (IFN-γ; 100 ug/ml) and actokine-2 (AK-2; 50 ug/ml) were provided by Actokine Therapeutics. AK-2 is a cytokine-based experimental antiviral being developed by Actokine Therapeutics, which consists of recombinant normal human proteins comprising part of the mammalian cell response to virus infection (Wong; unpublished). Soluble stocks of these agents were stored at 4°C in fetal bovine serum, which also served as the drug-vehicle control for the experiments, and were diluted 1:50 or 1:100 in medium to yield concentrations in cell cultures of about 1–2 ug/ml. As noted for individual experiments, cells were exposed to the drug or control treatments prior to PRRSV infection (pretreatment), during the course of PRRSV infection (delayed or post-treatment), or simultaneously with PRRSV infection. Based on previous studies of in vitro efficacy, the microtubule inhibitor colchicine which binds to tubulin (Sigma; 5 or 10 μM; [26–28]) or the microfilament disruptor cytochalasin D which depolymerizes actin (Sigma; 1 or 2 μM; [26,29-31]) were added to cell cultures at the times indicated.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

WAC conceived and designed the study, carried out experiments, performed data collection and analyses, and drafted the manuscript. RGD was responsible for cell culture, carried out some of the fluorescence analyses, and contributed to the FACS analyses. GHW prepared AK-2, IFN-γ, and control reagents and contributed to the experimental design. SS performed the FACS analyses. PWD carried out infection assays and performed some of the manual microscopic analyses. RRRR prepared pig macrophages, MARC-145 cells, and PRRSV for the project. EAN prepared the antibody reagent, MARC-145 cells, and PRRSV for the project. All authors made intellectual contributions to the study, and participated in the review and revision of the manuscript.

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